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## **3-Nitrotyrosine Predicts Healing in Chronic Diabetic Foot Wounds Treated with Hyperbaric Oxygen**

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### **INTRODUCTION**

The medical art of healing the chronic diabetic foot wound is to a great extent empirical. Current methods rely on visual examination of the wound. For example the appearance of granulation tissue and the formation of new epithelium are often noted as signs of positive treatment effect whereas increasing size of a wound may indicate treatment failure. Since changes in the chronic wound may occur over a period of several weeks, determining the effect of treatment can be problematic. Extensive documentation including photographs can contribute to the objectivity to this process. However extensive documentation adds to the time and cost of treatment and may not be feasible in many health care models. Wound treatment, including amputation, topical growth factors and HBO are also expensive. Finally, and most importantly, time and effort spent on ineffective treatment is time lost to the patient and perhaps a window of opportunity lost to the physician if the wound progresses and amputation is the only remaining treatment option. When these aspects of wound treatment and evaluation are considered, diagnostic methods that rapidly and objectively determine the effect of wound treatment are of considerable economic and therapeutic importance.

Our main hypothesis is that treatments that are effective will increase the production of NO in the chronic non-healing wound. In addition, increased NO production is an early event that could serve as a rapid indicator of treatment effect. In other words effective treatments will cause increased NO production whereas treatments that have little effect will not increase NO. There is significant evidence suggesting that NO does play an important role in healing of the chronic wound. NO is produced by a variety of cells including wound resident macrophages, epithelial cells, and neutrophils (1). NO promotes angiogenesis (2), keratinocyte proliferation (3), and collagen deposition (4,5,6), properties that are essential to wound repair. When other radicals such as superoxide are present, NO reacts to form an especially toxic radical, peroxynitrite, which is thought to be important in the reduction of bacterial colonization (7,8,9). NO has a half-life that is measured in seconds, however stable end-products of NO including nitrate and 3-NT have been used as an indirect measure of NO production.

The correlation between healing and increased NO production has been demonstrated in the non-healing diabetic wound. Non-healing diabetic wound exudate fluid nitrate levels have been shown to be decreased relative to wound exudate from surgical wounds of normal subjects suggesting that NO production is deficient in diabetics (10). In a recent report, we have shown that when non-healing diabetic foot wounds are treated with topical platelet derived growth factor (PDGF, Regranex), urine nitrate levels are 2-fold lower ( $P < 0.01$ ) for patients whose wounds still fail to heal compared to those whose wounds respond and heal (11). Plasma levels show a similar relationship with a four-fold difference on average though this difference was not found to be statistically significant due to inter-patient variance. These data show that increased NO production is correlated with wound healing and a favorable treatment outcome with topical PDGF. Taken together these results suggest that nitrate determination could be used to objectively evaluate the clinical response of the diabetic wound to treatment. However, systemic nitrate levels are problematic since substantial amounts of NO and consequently nitrate are produced during maintenance of vascular homeostasis and during disease processes characterized by inflammation. Thus systemic nitrate levels may be elevated in some patients, despite the absence of healing. In addition non-invasive sampling of wound exudate and subsequent determination of nitrate can be problematic if the wound produces little exudate. Another end-product of NO production, 3-NT, was therefore considered as a healing marker.

3-NT is produced *in situ* by the reaction of nitrating agents and free or protein associated tyrosine. Peroxynitrate is produced by the reaction of NO and superoxide, though other chemistries have also been

identified (12). The non-healing diabetic wound is composed macrophages and other immune effector cells that are inactive (13), but have the capacity to produce peroxynitrite. We hypothesized that an early event in healing is increased NO and consequently peroxynitrite, and as a result, increased cellular accumulation of 3-NT. In an earlier report we demonstrated that 3-NT accumulation occurs in activated murine macrophage-like cell line and that cellular accumulation can be measured using immunocytochemical methods in conjunction with flow cytometry (14). These previous findings suggested to us that cellular accumulation of 3-NT in cells collected from the wound could be used as an objective marker of healing during treatment with HBO.

## METHODS

### PATIENT SELECTION AND OUTCOME EVALUATION

Diabetic patients with non-healing wounds that had failed antibiotic therapy and meeting the criteria for treatment with HBO were enrolled in the study. Further, only those patients where the objective of treatment was wound healing were selected. In some cases HBO therapy is given to define viable tissue prior to amputation, thus in these cases healing is not the objective of therapy. In this study, subjects meeting these criteria were selected consecutively until a total of 12 total patients were acquired. One patient that was initially included was later excluded because he failed to return for treatments after initial evaluation. Patients inspired 100% oxygen for 90 minutes with 5 minute intervals of air every 30 minutes at a pressure of 2.4 atmospheres absolute once a day until their wound showed significant signs of healing or until treatment was determined to be ineffectual. A favorable outcome was determined by observing the formation of granulation tissue and re-epithelization of the wound bed, called herein progressive healing (PH), whereas those patients that showed little or no improvement during treatment are called herein minimal improvement (MI).

### TRANSCUTANEOUS OXIMETRY (TCOM)

Transcutaneous oximetry (TCOM) values were obtained on all patients prior to initiation of their HBO treatment series. Several TCM3 TCOM units by Radiometer (Copenhagen, Denmark) are currently used to gather this baseline data on all patients for our facility database. These values are obtained in the standard fashion after the skin sites are prepared by shaving, cleansing, and dabbing with adhesive tape. The monitor leads are attached after the ionic TCOM solution is placed in the membrane/ring electrode. The chest was used as the site for control values and the other values were obtained from skin near the wound.

### DETERMINATION OF 3-NT IN WOUND CELLS

Wound dressings were collected during wound care. Portions containing exudate were cut from the dressing and placed in 25ml of Dulbecco's phosphate buffered saline with a pH of 7.4 (DPBS), shaken for 3 minutes, and then centrifuged. The supernatant was aspirated and then the pellet was suspended in 10 ml DPBS. The cell suspension was then filtered through a 60  $\mu$ m nylon mesh to remove threads and other large debris. The filtered suspension was centrifuged, the supernatant aspirated, and the pellet suspended in fixative (FACS Lyse Solution, Beckton-Dickenson, San Jose, CA). Fixed wound exudate cells were washed twice in 10 ml DPBS and then blocked for non-specific binding of secondary antibody for 30 min with 10% normal goat serum. Cells were then suspended in 10  $\mu$ l of rabbit polyclonal IgG anti-3-NT antibody solution (Upstate Biotechnology, Lake Placid, NY), diluted 20-fold in DPBS in 10% normal goat serum, and incubated overnight in the dark at 4°C. Cells were then washed 2 times with 1 ml DPBS and suspended in 10  $\mu$ l of phycoerythrin-conjugated goat anti-rabbit Fab fragment solution (Sigma, ST. Louis, MO), diluted 20-fold in DPBS and then incubated for 1 hr at room temperature. Following incubation, the cells were suspended in 400  $\mu$ l DPBS. Processing for the 3-NT negative control was identical to the above with the exception that IgG collected from normal rabbit serum (DAKO, Cupertino, CA) was used instead of anti-3-NT IgG antibody. The concentration of rabbit IgG in the negative control was identical to anti-3-NT antibody. Flow cytometry was performed on a FACS Caliber instrument. The epithelial and PMN cells that we sought were separated from exudate debris using a dot-plot of linear side scatter and linear forward scatter of light, which

corresponded respectively to the size and roughness fingerprint of these known standard cell types. The identity of cells was confirmed by sorting the gated population and evaluating morphology using conventional light microscopy. The fluorescence intensity associated with phycoerythrin was measured using the peak height measured for each cell in the FL-2 band pass filter. The same instrument settings were used during all runs reported. The geometric mean of the population of FL-2 signals was computed using Cell Quest software (Becton-Dickinson) and used in subsequent statistical comparisons. A minimum of 10,000 cells was used for population statistics.

## RESULTS

Of the 12 patients considered in this study 6 showed progressive healing (PH) and 6 showed minimal improvement (MI) during treatment with HBO. Table 1 shows that demographic characteristics of PH and MI patients are similar. The table also shows that in all MI patients, neutrophils were recovered from the wound sample, whereas none were recovered from PH patients.

Our main hypothesis is that HBO increases the production of oxidative radicals and that this increase is essential for healing and will be revealed by increased cellular accumulation of 3-NT in healing wounds compared to non-healing wounds. In addition, we have postulated that production of oxidative radicals is a relatively early event in the healing process, thus cellular accumulation will decline in the later stages of healing. In contrast MI patients are expected to show no change in cellular accumulation of 3-NT during treatment with HBO. Figure 1 shows that the data collected during this study supports this hypothesis. PH and MI patients show similar levels of epithelial cell accumulation of 3-NT during the first week of HBO treatment, however by week two trends in accumulation diverge with PH patients showing significantly greater accumulation than MI patients. By week 5 3-NT levels decline in PH patients. Figure 2 shows that accumulation of 3-NT in epithelial cells is approximately 3 times greater in PH compared to MI patients ( $p < 0.001$ , Tukey-Kramer multiple comparison test)

Another hypothesis that has been addressed during the current investigation is that patients with lower Tcom's will respond less favorably to HBO. Table 2 shows that PH and MI patients are similar with respect to Tcom's. Correlation analysis of 3-NT accumulation and Tcom data did not reveal any association.

Table 1- Demographic, TCOM, cellular accumulation of 3-NT and outcome. Race: B=Black; W=white; H=Hispanic. Tcom values: first row are values when breathing air, chest reference/wound, second row when breathing 100% oxygen, chest reference/wound. Outcomes, PH- progressive healing; MI- minimal improvement.

Patient	Age	Race	Gender	Tcom Air C/W; O2 C/W	Cell Type Collected	Mean 3-NT (SD), Min, Max	Healing Outcome
A	62	W	M	85/89; 410/359	Epi	12.8 (9.1), 0.8, 22.2	PH
B	77	W	F	69/4; 324/69	Epi	25.7 (12.2), 15.3, 47.3	PH
C	63	H	M	83/29; 457/260	Epi	16.7 (10.5), 5.3, 37.8	PH
D	44	B	M	59/53; 330/113	Epi	15.8 (6.3), 2.9, 23.1	PH
E	72	W	M	63/47; 363/168	Epi	15.8 (16.0), -1.9, 49.3	PH
F	69	H	F	40/6; 355/16	Epi	3.5 (3.0), -1.0, 9.6	PH
G	63	W	M	54/35; 293/91	PMN	3.7 (1.1), 3.0, 4.5	MI
H	53	H	M	69/57; 384/109	Epi /PMN	5.9 (5.9), 0.9, 14.1	MI
I	58	B	M	37/0; 232/143	Epi /PMN	2.5 (3.2), -0.6, 5.7	MI
J	56	W	M	51/28; 420/140	Epi /PMN	10.5 (2.9), 7.8, 15.3	MI
K	73	H	M	79/60; 399/153	Epi /PMN	3.4 (1.5), 1.9, 5.7	MI
M	50	H	M	67/32; 477/229	Epi /PMN	4.5 (0.6), 3.8, 5.0	MI

Table 2- Relationship of Tcom values to outcome. Mean (SD).

Tcom	MI	PH
Chest, Air	60 (6)	67 (7)
Wound, Air	35 (9)	38 (13)
Chest, Oxygen	368 (36)	373 (21)
Wound, Oxygen	144 (19)	164 (52)

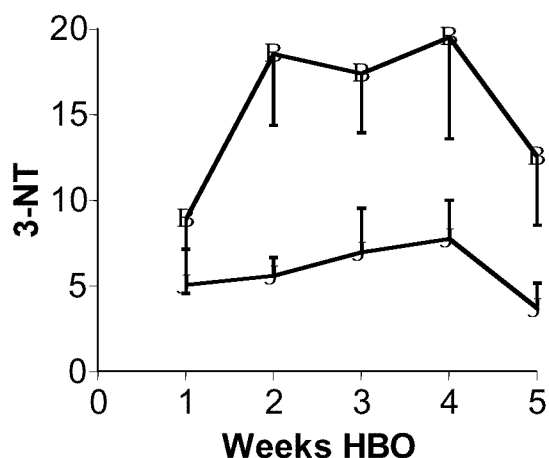


Figure 1- 3-NT in wound exudate cells in healing and non-healing patients. Black squares mean 3-NT of six PH patients during the period; Black circles mean 3-NT of six MI. Error bars are SEM.

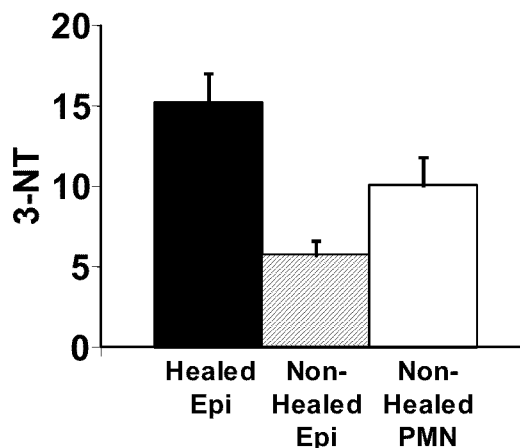


Figure 2- 3-NT cellular accumulation in healing and non-healing cells. PMN polymorphonuclear cells, i.e. neutrophils. Difference between epithelial cell accumulation of 3-NT in MI and PH patients highly significant, (Tukey-Kramer Honest Significant Difference test,  $p < 0.001$ ) other differences not significant.

## CONCLUSIONS

A non-healing wound in a patient with diabetes requires aggressive intervention to prevent the loss of a limb (15). HBO treatment is an approved intervention that has been shown to be effective (16) and is sometimes used as an adjunct to antibiotics (17). Since the cost of HBO treatment is high, \$14,000 for 30 treatments at one treatment center (18), diagnostic methods that reduce treatment time and cost are beneficial. We reasoned that a biomarker linked to early events during healing could be used to determine if a patient is responding to treatment, or if another treatment modality should be employed. Early determination of treatment effect would result in improved resource management and improved patient care.

Although patient numbers are small in this study, some clear trends are observed. Identification of neutrophils in the cell suspension obtained from the wound predicts unfavorable treatment outcome with HBO. Since neutrophils are associated with the acute response to bacterial infection one explanation is that antibiotic therapy was insufficient and that normal healing could therefore not proceed. HBO has been shown to selectively inhibit neutrophil adhesion (19) in healthy subjects, thus another possible explanation is

that in these diabetic subjects, neutrophil response was inappropriate leading ultimately to wound failure. Both explanations deserve additional investigation.

3-NT accumulation in epithelial cells is increased in PH but not MI patients during treatment with HBO. This supports our primary hypothesis that increased oxygen partial pressure in the wound will lead to increased production of oxidative radicals such as peroxynitrite that in turn nitrate proteins, including those found in epithelial cells. In all PH patients epithelial cell accumulation of 3-NT was near 0 at the beginning of HBO treatment and then increased by at least 3 fold during the next several weeks. We did not find any relationship between Tcom values and wound healing outcome or 3-NT accumulation suggesting that oxygen increase above some threshold is sufficient to cause increased production of nitrating radicals and also healing. These findings suggest that changes in epithelial cell accumulation of 3-NT could be used to determine if patients are responding to HBO treatment. Patients that fail to show an increase in 3-NT during the first 2 weeks of treatment should receive another treatment besides HBO.

The chemical kinetics governing the accumulation of 3-NT in wound exudate cells is unknown. Both epithelial cells and neutrophils are capable of producing nitrating species. However the processes governing the accumulation of nitro adducts, including 3-NT, in cells is only poorly understood. Recently denitrase-like activity has been identified in some organs (20) and hypochlorous acid produced by neutrophils may cause the removal of the nitro group from 3-NT (21). There is evidence that HBO inhibits transcription of inducible nitric oxide synthase gene in murine peritoneal macrophages suggesting indirectly that HBO may inhibit formation nitrating species (22). Clearly more research is required to definitively identify the mechanisms which govern 3-NT accumulation. Cellular accumulation occurs when the rate at which adducts are formed exceeds the rate at which they are removed. We have hypothesized that in the healing wound production of nitric oxide increases leading to increased nitrating species and increased nitro adduct formation. Given our current understanding of the chronic non-healing diabetic wound it appears that increased oxygen partial pressure can facilitate increased nitric oxide synthesis rate and in turn increase healing rate.

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